JOURNAL OF VIROLOGY, Mar. 1999, p. 2469–2480 Vol. 73, No. 3
0022-538X/99/$04.00+0


# A Conserved Tryptophan-Rich Motif in the Membrane-Proximal Region of the Human Immunodeficiency Virus Type 1 gp41 Ectodomain Is Important for Env-Mediated Fusion and Virus Infectivity

KARL SALZWEDEL,† JOHN T. WEST, AND ERIC HUNTER*

*Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294*

Received 10 August 1998/Accepted 9 December 1998

**Mutations were introduced into the ectodomain of the human immunodeficiency virus type 1 (HIV-1) transmembrane envelope glycoprotein, gp41, within a region immediately adjacent to the membrane-spanning domain. This region, which is predicted to form an α-helix, contains highly conserved hydrophobic residues and is unusually rich in tryptophan residues. In addition, this domain overlaps the epitope of a neutralizing monoclonal antibody, 2F5, as well as the sequence corresponding to a peptide, DP-178, shown to potently neutralize virus. Site-directed mutagenesis was used to create deletions, substitutions, and insertions centered around a stretch of 17 hydrophobic and uncharged amino acids (residues 666 to 682 of the HXB2 strain of HIV-1) in order to determine the role of this region in the maturation and function of the envelope glycoprotein. Deletion of the entire stretch of 17 amino acids abrogated the ability of the envelope glycoprotein to mediate both cell-cell fusion and virus entry without affecting the normal maturation, transport, or CD4-binding ability of the protein. This phenotype was also demonstrated by substituting alanine residues for three of the five tryptophan residues within this sequence. Smaller deletions, as well as multiple amino acid substitutions, were also found to inhibit but not block cell-cell fusion. These results demonstrate the crucial role of a tryptophan-rich motif in gp41 during a post-CD4-binding step of glycoprotein-mediated fusion. The basis for the invariant nature of the tryptophans, however, appears to be at the level of glycoprotein incorporation into virions. Even the substitution of phenylalanine for a single tryptophan residue was sufficient to reduce Env incorporation and drop the efficiency of virus entry approximately 10-fold, despite the fact that the same mutation had no significant effect on syncytium formation.**

The process of viral entry is a key step in the initiation of human immunodeficiency virus type 1 (HIV-1) infection. Attachment of the HIV-1 virion to the target cell is mediated by binding of the viral envelope glycoprotein complex to the CD4 receptor on the cell surface (reviewed in reference 49). The envelope glycoprotein (Env) is processed from an oligomeric precursor protein, gp160, into two noncovalently linked subunits: the surface subunit, gp120, which is responsible for binding to CD4, and the transmembrane subunit, gp41, which initiates membrane fusion. In addition to CD4, HIV-1 has also been shown to require an interaction between gp120 and a coreceptor, members of a family of seven-transmembrane G protein-coupled chemokine receptors, on the cell surface in order to mediate membrane fusion (18). Many viruses, such as the influenza virus, are endocytosed following binding to their receptor (35). Their viral glycoproteins then undergo a pH-activated conformational change to a fusion-competent form in the acidic environment of the endosome (13, 23). HIV, however, fuses directly with the plasma membrane at the surface of the target cell in a pH-independent manner (37, 38, 53). In the case of HIV, it has been shown that binding to CD4 induces conformational changes in gp120 and increases the exposure of gp41 epitopes independent of gp120 dissociation (48, 50, 51). Thus, it has been proposed that the envelope glycoprotein of HIV undergoes receptor-induced fusion activation analogous to the pH-induced activation of other viral glycoproteins.

The amino-terminal fusion peptide of gp41 was originally identified by its similarity to functional domains of paramyxovirus glycoproteins (22) and has since been extensively characterized through mutagenesis as the primary fusion domain (3, 19, 52). Based on analogy to similar rearrangements in the glycoproteins of influenza virus (13) and Rous sarcoma virus (24, 27), the CD4-induced conformational change in the HIV-1 glycoprotein is believed ultimately to lead to the insertion of the fusion peptide into the target cell membrane. The details of this conformational change and how the gp120-coreceptor interaction contributes to it are unclear. However, recent studies have suggested that domains of gp41 may also be involved in a conformational change leading to fusion. Mutagenesis of a heptad repeat, leucine zipper-like motif in the ectodomain of gp41 demonstrated that the hydrophobicity of position 573 in the center of the repeat dictated the efficiency with which gp41 mediated fusion (9, 10, 16). It has been hypothesized, based on peptide studies (7, 55–60), that this motif is involved in forming a coiled-coil structure in the glycoprotein oligomer, similar to that formed by an acid-activated fragment of hemagglutinin (HA) (5), following receptor binding.

While it has been shown that the cytoplasmic tail of gp41 is not required for fusion (17, 21, 62, 63), we and others have shown that the fusion event does require that gp41 contain a membrane-spanning peptide anchor, since substitution of a covalently linked lipid anchor for the membrane-spanning do-

* Corresponding author. Mailing address: Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294. Phone: (205) 934-4321. Fax: (205) 934-1640. E-mail: ehunter@uab.edu.

† Present address: Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892.

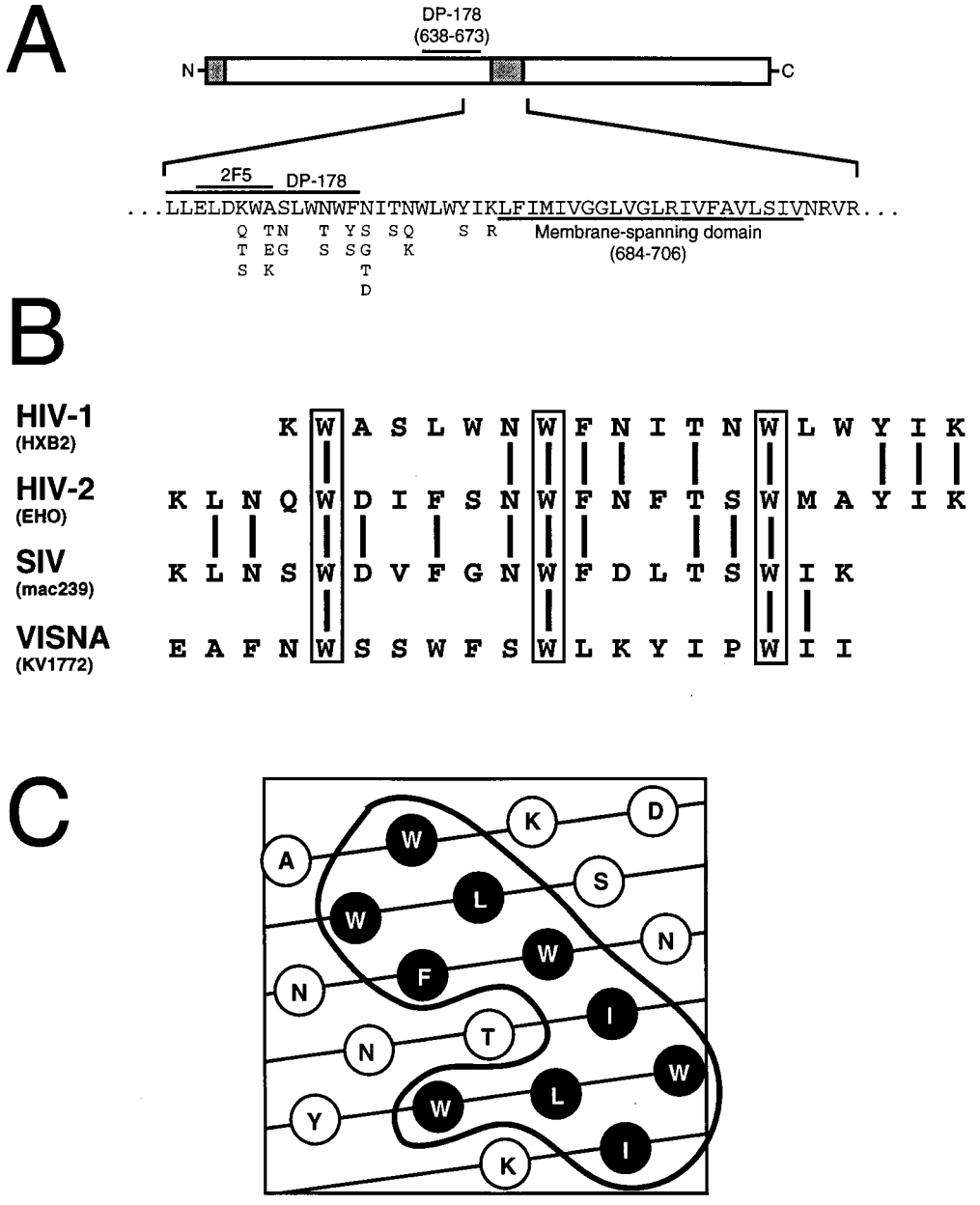


FIG. 1. Sequence conservation and predicted structure of the tryptophan-rich region. (A) Schematic representation of gp41, showing the location and sequence variability of the tryptophan-rich region and the overlapping sequences of the 2F5 epitope and DP-178 peptide. Shaded regions indicate the N-terminal fusion peptide and the membrane-spanning domain. The amino acids in the HIV-1 sequence database which occur at each position are denoted underneath the respective amino acid in the HXB2 strain. (B) Alignment of the tryptophan-rich sequence of HIV-1 HXB2 gp41 with homologous regions of HIV-2, SIV, and visna virus glycoproteins. Conserved tryptophan residues are boxed. (C) Helical net projection of the tryptophan-rich region. The outlined region indicates the cluster of conserved hydrophobic residues shown in black.

main of gp41 did not support fusion (46, 54). The structural requirements of this membrane-spanning domain, however, are less clear. Truncation of the gp41 carboxy terminus beyond the lysine residue at position 683 (HXB2 strain) is sufficient to cause a loss of membrane anchoring and secretion of the glycoprotein in a soluble form (17, 43), whereas truncation to just after the arginine at position 696 results in a stably anchored protein that is expressed on the cell surface (43), suggesting that the minimal membrane-spanning domain could be residues 684 to 695. Similar results have been obtained for the simian immunodeficiency virus (SIV) Env proteins where residues 691 to 707 are sufficient to anchor the protein in the membrane and mediate incorporation into virions (29). However, these truncated glycoproteins were nonfunctional, and residues C terminal to this minimal anchor domain are important for the function of the glycoprotein (43). These results suggest that while the glycoprotein can be forced to use an abbreviated membrane-spanning domain, such a domain is not sufficient for the completion of the fusion reaction.

The studies described here focus on a membrane-proximal region in the ectodomain of gp41 that is unusually rich in tryptophan residues, several of which are conserved in both

primate and ungulate lentiviruses (Fig. 1A and B). This region overlaps a biologically important domain of gp41. Two peptides, DP-178 and SJ-2176, derived from this membrane-proximal region of gp41 have been shown to potently inhibit HIV-mediated fusion (28, 61). A neutralizing human monoclonal antibody, 2F5, has also been described whose epitope overlaps the C terminus of the DP-178 peptide (40, 41). Interestingly, this epitope has been shown to become inaccessible following CD4 binding, suggesting that this region may undergo a change in conformation (51).

In order to examine the role of the tryptophan-rich membrane-proximal domain of the HIV-1 gp41 ectodomain in the structure and function of the envelope glycoprotein, we have created deletion, substitution, and insertion mutations in this region by site-directed mutagenesis. Our analyses of the resulting mutant glycoproteins indicate that a stretch of 17 hydrophobic and uncharged amino acids immediately adjacent to the border of the membrane-spanning domain is dispensable for the normal maturation, transport, and CD4-binding ability of the protein but is required for Env-mediated membrane fusion. In contrast to the deletions and multiple point mutations required to abrogate fusion, even subtle mutations of the conserved tryptophan residues had a profound effect on incorporation of the mutant glycoproteins into virions and on virus infectivity. Features of the structure and function of this tryptophan-rich motif are discussed in relation to its possible function in the fusion mechanism and glycoprotein incorporation into virus.

## MATERIALS AND METHODS

**Cell culture.** COS-1 and 293T cells were obtained from the American Type Culture Collection. HeLa-CD4/LTR-β-gal cell lines were obtained through the AIDS Reference and Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and were originally contributed by Michael Emerman. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. HeLa-CD4/LTR-β-gal cells were additionally maintained in medium containing hygromycin and G418 (Geneticin) as recommended by the contributor.

**Mutagenesis and construction of plasmids.** Construction of the AK1 and AK2 mutants has been described previously (46). To create the AΔK1-K2 mutation, constructs containing either the AK1 or AK2 mutation were digested with *Ava*I, and the resulting fragments of the two constructs were ligated to one another in such a way as to delete the intervening sequence between the two mutations. The +FLAG and +DAF mutations were created by site-directed mutagenesis with the Altered Sites system from Promega.

To create simpler mutations in the tryptophan-rich region, an *env* expression plasmid was first modified by site-directed mutagenesis to encode two unique restriction enzyme sites within or adjacent to the coding sequence for the tryptophan-rich region. pSRHS, a simian virus 40 (SV40)-based expression vector, was first digested with *Xba*I, filled in by using the Klenow fragment of DNA polymerase to create a blunt end, and religated, destroying an *Xba*I site in the polylinker. An oligonucleotide was then designed which encoded both an *Xba*I site and an *Nhe*I site for use in the Altered Sites system to create an expression plasmid, pSRHS-XUN, containing a unique *Xba*I site (HXB2 nucleotide 7750) adjacent to and an *Nhe*I site (nucleotide 7769) within the coding sequence for the tryptophan-rich region while retaining the wild-type peptide sequence. The Altered Sites system was then used to destroy a second *Nhe*I site in the HXB2 *env* gene (nucleotide 6806) without affecting the peptide sequence. The final plasmid, pSRHS-XNU, and the corresponding *env* fragment served as the wild-type control used in this study.

A reverse PCR primer which overlapped a unique 3′ *Bam*HI site in the gp41 coding sequence was designed to amplify the noncoding strand. This primer was used in combination with mutagenic primers overlapping either the unique *Xba*I or *Nhe*I site to create *Xba*I-*Bam*HI or *Nhe*I-*Bam*HI fragments, respectively, which were then substituted into the pSRHS-XNU plasmid. Certain constructs containing multiple mutations were generated by using a primer encoding a secondary mutation to prime PCR from a previously mutated construct. Likewise, some mutated PCR products were substituted via the *Nhe*I and *Bam*HI sites into constructs containing upstream mutations to create multiple mutations. A primer overlapping the unique *Xba*I site was used with the *Bam*HI reverse primer to amplify the mutated fragment for insertion into pSRHS-XNU. All mutations were confirmed by DNA sequencing with a primer approximately 100 bp upstream of the coding sequence for the tryptophan-rich region to read from upstream of the mutagenic primer through the coding sequence of the membrane-spanning domain. The phenotypes of a majority of the mutated constructs were confirmed by comparison of two independent clones.

For construction of proviral clones, an *Nhe*I-*Bam*HI HXB2 *env* fragment, resulting from a partial digest of the original pSRHS-XUN construct created by using the *Nhe*I site at nucleotide 6806 and containing the engineered *Xba*I and *Nhe*I sites, was substituted into the *env* gene of the pNL4-3 proviral clone. The resulting clone, pNL4-3/XUN, was used as a positive control. *Xba*I-*Bam*HI fragments derived from pSRHS-XNU mutant *env* constructs were then substituted into pNL4-3/XUN. All proviral constructs were confirmed by sequencing as described above.

**Protein expression and radioimmunoprecipitation.** SV40-based *env* expression plasmids were transfected into COS-1 cells in 60-mm-diameter plates by using DEAE-dextran (1 mg/ml). At 36 to 48 h posttransfection, the cells were starved in methionine- and cysteine-free DMEM for 20 min and pulse-labeled in methionine-cysteine-free DMEM supplemented with [$^{35}$S]methionine-cysteine (357 μCi/ml; DuPont-NEN) for 30 min. The labeled cells were then chased in complete DMEM for 3 h prior to harvest of the medium and lysis of the cells. Cells were lysed by a 10-min incubation on ice in lysis buffer (1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], and 0.5% sodium deoxycholate, in phosphate-buffered saline [PBS]). Cellular debris was removed by centrifugation in a microcentrifuge for 5 min at 4°C. HIV-1 glycoproteins were immunoprecipitated from the cell lysate (C) and medium (M) by incubation for 1 h at 4°C with AIDS patient serum. The immune complexes were incubated for 30 min at room temperature with fixed *Staphylococcus aureus* cells (Staph A) and pelleted in a microcentrifuge. The pellets were washed three times in lysis buffer lacking 0.5% sodium deoxycholate and once in 20 mM Tris-HCl (pH 6.8) and were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

**CD4 binding assay.** Following pulse-chase labeling of transfected COS-1 cells as described above, cells were lysed in lysis buffer lacking SDS. The cell lysate was clarified by centrifugation and divided into two tubes. One-half of the lysate was incubated with patient serum as described above. The other half was incubated overnight with 0.5 μg of CD4-immunoglobulin G (IgG) (Genentech; kindly provided by Mark Mulligan [University of Alabama at Birmingham]) at 4°C. Both samples were immunoprecipitated with Staph A and analyzed as described above.

**Cell surface biotinylation.** Glycoproteins on the cell surface were biotinylated based on the method of Lisanti et al. (34). Following pulse-chase radiolabeling as described above, transfected COS-1 cells were placed on ice in a cold room and washed three times with ice-cold PBS containing calcium and magnesium (PBS-C/M). The cells were then incubated with PBS-C/M containing 0.5 mg of NHS-SS-biotin per ml for 30 min on ice. The reaction was quenched by incubation of the cells in ice-cold serum-free DMEM for 10 min on ice. The cells were then washed twice with ice-cold PBS-C/M containing 20 mM glycine and lysed in lysis buffer containing 20 mM glycine. Following immunoprecipitation with patient serum as described above, the Staph A pellet was resuspended in PBS containing 2% SDS and boiled for 5 min. The sample was then diluted with PBS to a final concentration of 0.05% SDS and incubated with 40 μl of a 50% slurry of streptavidin-agarose beads (Pierce) at 4°C overnight. The beads were then pelleted, washed, and prepared for SDS-PAGE as described above for immunoprecipitates.

**Cell-cell fusion assays.** Twenty-four hours posttransfection, COS-1 cells in a six-well plate were trypsinized, mixed approximately 1:10 with untransfected HeLa-CD4/LTR-β-gal indicator cells, and replated in a six-well plate. Twenty-four hours after replating, fusion of the two cell types was detected microscopically following staining in situ with 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-Gal) as described previously (31).

**Virus expression and pelleting.** Virus expression was assayed by labeling COS-1 cells transfected with pNL4-3 proviral clones, followed by lysis, immunoprecipitation, and SDS-PAGE as described above for the analysis of glycoprotein expression.

For virus pelleting, 293T cells in 35-mm-diameter plates were transfected with pNL4-3 proviral clones by a modified calcium phosphate technique (8). Forty-eight hours after DNA was added to the cells, the cells were metabolically radiolabeled as described above for 30 min and chased for 20 h in 1 ml of complete growth medium. Culture supernatants were then collected and filtered through a 0.45-μm-pore-size filter to remove cellular debris. The filtered supernatants were then transferred to a microcentrifuge tube, underlaid with a 20-μl sucrose cushion (10% sucrose in PBS), and centrifuged for 4 h at room temperature in a microcentrifuge to pellet virions. The supernatant was then carefully and completely removed, and the virus pellets were lysed for 10 min at room temperature prior to immunoprecipitation with patient serum as described above and SDS-PAGE.

**Virus entry assay.** Sixty to seventy-two hours after transfecting 293T cells as described for virus pelleting, culture supernatants were collected and filtered through a 0.45-μm-pore-size filter. Relative levels of reverse transcriptase activity were determined for each sample as previously described (16), and the volumes were normalized by dilution with complete medium. The normalized supernatants were used to infect HeLa-CD4/LTR-β-gal cells in duplicate as described previously by Kimpton and Emerman (31). Supernatants were diluted as necessary to remain within the quantifiable range of the assay.

## RESULTS

**Mutagenesis of the membrane-proximal region of the HIV-1 transmembrane glycoprotein.** To examine the functional role of the tryptophan-rich membrane-proximal region of gp41, deletion, substitution, and insertion mutations were created (Fig. 2, left). Two mutations, AK1 and AK2, were designed previously which created *Ava*I sites at the coding sequences for the lysines at either the N-terminal (AK1) or C-terminal (AK2) border of the 17-amino-acid tryptophan-rich region (46). These mutations substitute an Asn-Ser-Gly peptide sequence at either end of the tryptophan-rich sequence. In order to determine if the tryptophan-rich region of gp41 was necessary for fusion, a preliminary mutant, AΔK1-K2, was constructed which contains a deletion of 18 residues, including the entire tryptophan-rich sequence.

More specific deletions (Δ665–682, Δ666–670, Δ671–677, Δ678–682, and Δ666–670/678–682) were then constructed to probe for critical subdomains within this region. In addition, two mutations were specifically designed to probe the structural requirements of the region. SC7 scrambles the central seven residues, 671 to 677, in a nonconservative substitution pattern, switching the highly conserved hydrophobic positions with the nonconserved neutral and polar positions (Fig. 1A). ΔFN deletes two central nonconserved residues, Phe 673 and Asn 674, in an attempt to disrupt the alignment of a potential α-helical structure (Fig. 1C).

Single amino acid substitutions were made for each of the conserved tryptophan residues to determine their roles in the function of the membrane-proximal region. Alanine was substituted for the first (W666A), second (W670A), fourth (W678A), and fifth (W680A) tryptophan residues within this 17-amino-acid stretch. Serine, proline, and phenylalanine were all substituted for the third tryptophan (W672S, W672P, and W672F, respectively). In addition, multiple substitutions were combined to further define the sequence requirements of the region. Multiple substitutions of alanine for tryptophan residues are denoted by parenthetically listing the relative positions of the changed tryptophans within the membrane-proximal region. For example, W(2,3)A changes the second and third tryptophan residues to alanines. One of these multiple substitutions, W(1,3,4)A, changes the three tryptophan residues that are conserved in HIV-2 and SIV (Fig. 1B).

Finally two insertion mutants were generated. +FLAG introduces six highly charged residues into the epitope of the 2F5 neutralizing monoclonal antibody in such a way as to create the eight-amino-acid FLAG epitope (Kodak-IBI). The other insertion mutant, +DAF, was designed to introduce the coding sequence of the nine membrane-proximal residues of human erythrocyte decay-accelerating factor (DAF) between the tryptophan-rich region and the membrane-spanning domain.

**Expression of envelope glycoprotein mutants.** Each of the plasmids containing mutated *env* genes was transiently expressed in transfected COS-1 cells. The proteins were metabolically labeled in a pulse-chase experiment, and the resulting products were immunoprecipitated from both the cell lysates and the cell medium (Fig. 3). All of the mutant glycoprotein products were expressed at levels similar to those of the wild-type protein and were processed normally to gp120 and gp41, with the sole exception of the initial deletion, AΔK1-K2. This mutation was found to significantly reduce the efficiency with which the glycoprotein precursor was proteolytically processed (Fig. 3, AΔK1-K2, lanes C and M). Processing of the Δ665–682 mutant glycoprotein, however, was not significantly affected (Fig. 3, Δ665–682, lanes C and M). Quantitation of the proportion of gp120 found in the medium relative to cell-associated gp120 revealed no significant effects of the mutations on gp120-gp41 association relative to wild-type Env (data not shown).

All of the protein products were of the predicted size, based on the presence or absence of deleted residues, suggesting that the mutant glycoproteins were glycosylated normally. No variation in apparent molecular weight was detected among mutations which disrupted the potential signal for N glycosylation at residue 674. This is consistent with a previous report demonstrating that this nonconserved signal was not utilized (32). One exception, the gp41 subunit of the SC7 mutant, migrated more rapidly than was expected (Fig. 3, SC7, lane C). Since this mutation did not delete any residues, it appears that the scrambling of the peptide sequence was sufficient to alter the mobility of the subunit in the SDS gel. Similar departures from the predicted mobility have been noted previously with gp41 when predicted amphipathic helical regions within the cytoplasmic tail were lost upon truncation of the protein (17, 46). Since the SC7 mutation was designed specifically to disrupt the central predicted amphipathic helical region of the tryptophan-rich region (Fig. 1C), it seems reasonable that the predicted migration of this mutant was altered in a fashion similar to that of the truncation mutants.

Previous reports have demonstrated that the tryptophan-rich region is not sufficient to anchor the glycoprotein in the membrane (17, 43). All of the cell cultures expressing mutant glycoproteins, including the AΔK1-K2 and Δ665–682 mutants, released gp120, but not gp41 or gp160, into the culture medium (Fig. 3, M lanes), indicating that the mutant glycoprotein precursors and transmembrane subunits were stably anchored in the membrane. This demonstrates further that the membrane-proximal region is not only insufficient but is also unnecessary for the stable association of the glycoprotein with the membrane. The results presented above demonstrate that the tryptophan-rich region is not required for the normal biosynthesis and maturation of the glycoprotein.

**Effects of the mutations on cell-cell fusion.** In order to assess the fusogenicity of different constructs, we have found that quantitation of the average number of nuclei per syncytium, rather than the number of syncytia, typically provides a more consistent and reproducible measure of this biological activity. For this study, we used a highly sensitive approach to assay cell-cell fusion. COS-1 cells expressing the mutant glycoproteins as demonstrated in the previous experiments were mixed with HeLa-CD4/LTR-β-gal (MAGI) cells at a ratio of approximately 1:10. Because the *env* expression plasmids also express the viral Tat transcriptional activator protein, fusion of the two cell types induces the expression of a β-galactosidase reporter gene under control of the viral long terminal repeat (LTR) promoter (31). Because the β-galactosidase has been modified to contain a nuclear targeting signal, the nuclei of the resulting syncytium stain a dark blue with X-Gal in situ (31). This simplifies the microscopic quantitation of both the average number of syncytia in a low-power field and the average number of nuclei per syncytium. Immunofluorescent staining of selected cultures scoring negative for fusion in this assay demonstrated that individual COS-1 cells were indeed expressing high levels of glycoprotein but were failing to fuse with the MAGI cells (data not shown).

The AK1 mutant was found to reduce fusogenicity approximately 25%, despite the substitution of three residues within the epitope for the neutralizing monoclonal antibody, 2F5 (Fig. 2, right panel). This suggests that the neutralizing ability of 2F5 is not due to the masking of critical residues within its epitope. AK2, however, had a more significant effect on fusion, yielding only about 30% of the wild-type level of fusion. The

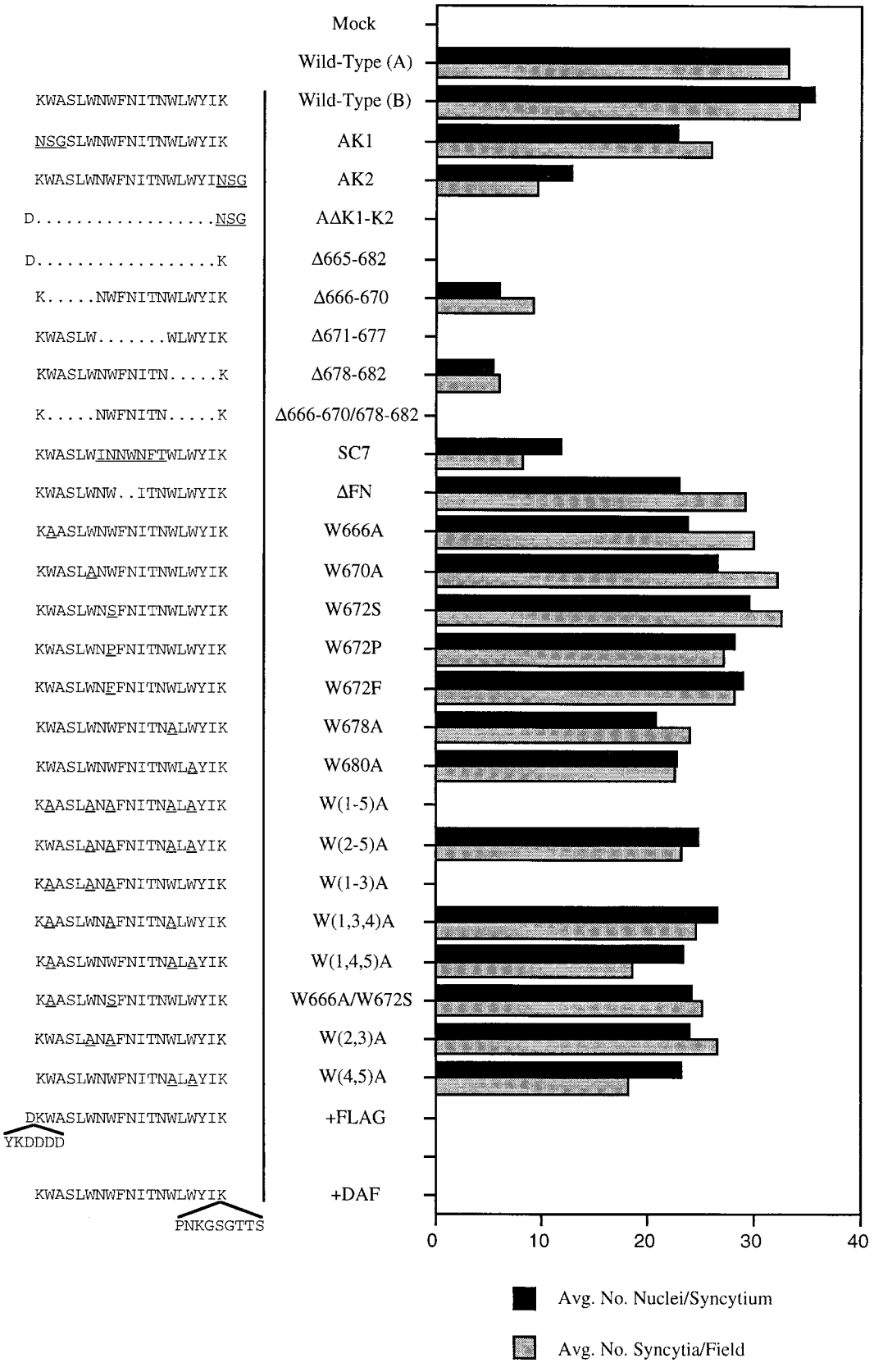


FIG. 2. Diagram of mutations and their corresponding fusogenicities. The Env mutations within the tryptophan-rich region are diagrammed in the left panel, juxtaposed to the corresponding name of the mutant and the cell-cell fusion data from a representative experiment. Amino acid changes are underlined, and deletions are denoted by periods. COS-1 cells expressing glycoprotein were mixed 1:10 with HeLa-CD4/LTR-β-gal cells and replated. The cells were stained 24 h later with X-Gal, and blue syncytia were quantitated microscopically. The average number of syncytia per low-power field was determined by counting six nonoverlapping fields per well for each of two wells and averaging the total. The average number of nuclei per syncytium was determined by quantitating 15 syncytia in each of two wells and averaging the total. Wild-Type (A) and Wild-Type (B) indicate results from two separately prepared plasmid clones.

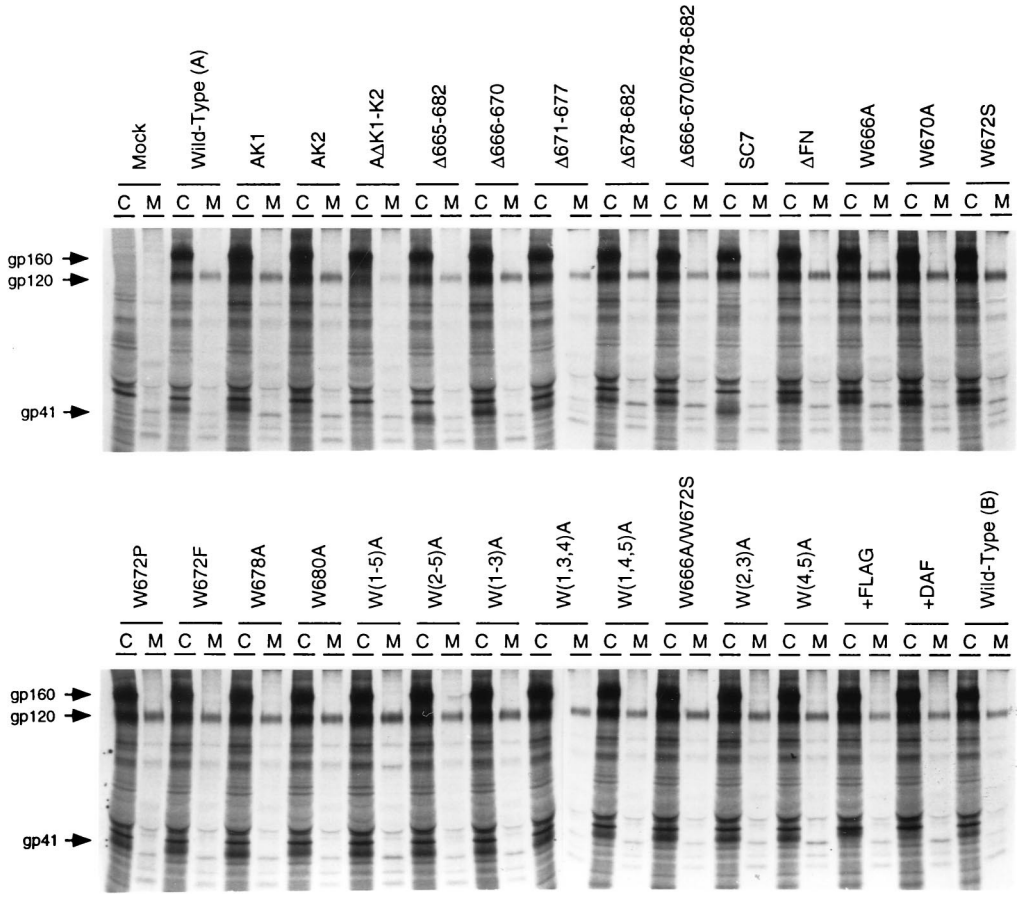


FIG. 3. Expression and maturation of envelope glycoprotein mutants. SV40-based *env* expression plasmids were transfected into COS-1 cells. Cells were labeled with [$^{35}$S]methionine-cysteine, and HIV-1 glycoproteins were immunoprecipitated as described in Materials and Methods from the cell lysate (C) or culture medium (M) by using AIDS patient serum for analysis by SDS-PAGE (8% polyacrylamide). Mock, transfected with wild-type *env* plasmid lacking a eukaryotic transcriptional promoter.

deletion mutant AΔK1-K2 was found to be completely defective in mediating fusion. Although this mutant was processed less efficiently than the other mutants, it is unlikely that the reduced level of processed glycoprotein, alone, was sufficient to block fusion in this assay.

A more precise deletion of the entire tryptophan-rich region (Δ665–682), as well as a deletion of the central seven amino acid residues, alone (Δ671–677), was sufficient to abrogate cell-cell fusion, demonstrating conclusively that the tryptophan-rich region is indeed critical for the fusion function of the glycoprotein. Deletion of either the first or last five residues of the region (Δ666–670 or Δ678–682, respectively) resulted in an approximately 80% reduction in fusogenicity as measured by both the average number of syncytia and the average number of nuclei per syncytium. Since the central seven residues were found to be necessary for membrane fusion to occur, the deletions of the first and last five residues were combined to address whether the central seven residues were also sufficient for fusion. The resulting mutation, Δ666–670/678–682, also abrogated fusion, indicating that the central seven residues of this sequence are necessary but not sufficient to mediate the function of this region in fusion. This suggested that multiple residues contribute to the function of this region.

To distinguish between a requirement for the specific sequence of the central portion of this region and simply a requirement for a certain number of residues, the central seven amino acid residues were scrambled. Because this sequence is predicted to form an amphipathic α-helix involving highly conserved hydrophobic residues (Fig. 1C), particular effort was made to scramble the sequence such that the positions of hydrophobic and polar residues were exchanged. The resulting mutation, SC7, resulted in an almost fourfold reduction in fusogenicity, indicating that there was some specificity for the sequence of residues, but that this specificity was not absolute for the functional role of the region.

To further test the potential role of an α-helical structure in the function of this region, two central nonconserved residues were deleted. The rationale was that such a deletion in the middle of a helix would skew the structure such that residues that previously aligned on one face of the helix would become displaced. This mutation, ΔFN, was found to have no significant effect on fusion, arguing against a required helical organization of the tryptophan residues.

Because the most striking feature of this region of the glycoprotein is the unusually high density of tryptophan residues, all of which are highly conserved in all HIV-1 isolates, single amino acid substitutions were made at each of these five positions. Surprisingly, none of these substitutions had any significant effect on fusion. None were as fusogenic as the wild type, but the greatest effect of any one mutation was only a 25% reduction in fusion (W680A). While most of the tryptophans were replaced only with alanine, the central tryptophan

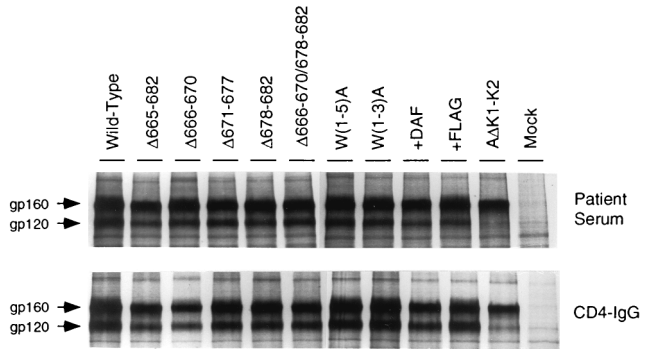


FIG. 4. CD4-binding capability of envelope glycoprotein mutants. Radiolabelled HIV-1 glycoproteins were immunoprecipitated from one-half of the cell lysate with CD4-IgG (bottom panel) and from the other half of the lysate with patient serum (top panel). The immunoprecipitates were analyzed by SDS-PAGE (8% polyacrylamide).

was substituted for with serine, proline, and phenylalanine. The lack of a major effect caused by the proline substitution provided further evidence against the requirement for a rigid α-helical structure in this region.

Since single amino acid substitutions had only a limited effect on fusion, multiple mutations were combined and assayed for an effect. Substitution of alanine for all five of the tryptophan residues together [W(1–5)A] was sufficient to abrogate fusion, demonstrating that the function of this region is indeed dependent on the highly conserved tryptophan residues. Surprisingly, however, the replacement of even a single tryptophan [W(2–5)A] was sufficient to return fusion to approximately 70% of the wild-type level. Even more surprising was the abrogation of fusion when just the first three tryptophans were replaced [W(1–3)A]. The other combinations of substitutions were only slightly less fusogenic than the single amino acid substitutions. This includes the W(1,3,4)A mutation, which changed the three tryptophans that are also conserved in HIV-2 and SIV (Fig. 1B).

Finally, two insertion mutations, +FLAG and +DAF, were found to completely abrogate fusion. The +FLAG mutation inserts six residues, five of which are charged, between two charged residues within the epitope for 2F5. Although this region is accessible to the 2F5 antibody and is relatively refractory to substitution mutations (AK1 and W666A) as well as deletions (Δ666–670), these results indicate that it cannot accommodate amino acid insertions and still maintain function.

The +DAF mutation, which inserts nine residues from the human DAF protein between the tryptophan-rich region and the membrane-spanning domain, also completely abrogated syncytium formation. The insertion of a similar peptide into the analogous position in influenza virus HA did not affect fusion (30), and so it is likely that the tryptophan-rich region must function in close proximity to the membrane to induce syncytium formation.

**Ability of the mutant glycoproteins to bind CD4.** To determine whether mutations in the tryptophan-rich region affected the ability of the glycoprotein to bind CD4, selected mutant glycoproteins which exhibited reduced levels of fusion or a lack of fusion were immunoprecipitated from metabolically radiolabeled COS-1 cells with CD4-IgG in parallel with patient serum (Fig. 4). All of the mutant glycoproteins bound CD4-IgG at levels similar to those of wild-type glycoprotein, indicating that the membrane-proximal region was dispensable for the efficient binding of the glycoprotein to its primary receptor.

**Transport of the mutant glycoproteins to the cell surface.** Because the ability of the mutant glycoproteins to form syncytia could be affected by reduced cell surface expression, it was necessary to determine the relative levels of each of the fusion-defective mutant glycoproteins on the surface of transfected cells compared to the level of wild-type glycoprotein. Metabolically radiolabeled cells expressing mutant glycoproteins were treated on ice with the membrane-impermeable, thiol-cleavable biotinylation reagent NHS-SS-biotin. Viral glycoproteins were initially immunoprecipitated from the cell lysate of transfected COS-1 cells by using HIV-positive patient serum to isolate the viral protein from cellular proteins. The immune complexes were then denatured by being boiled in SDS, and a portion of this immunoprecipitate was reserved for analysis in parallel with subsequent samples. The remaining sample was incubated a second time with streptavidin-agarose to isolate just the subpopulation of viral glycoproteins which were exposed to biotinylation reagent on the cell surface.

As can be seen in Fig. 5, all of the fusion-defective glycoprotein mutants except AΔK1-K2, which was also found to be processed inefficiently, were detected on the cell surface at levels similar to wild-type glycoprotein. To control for biotinylation of only glycoproteins that were expressed on the cell surface, a modified HIV-1 glycoprotein containing an endoplasmic reticulum retrieval signal attached to the C-terminus of its cytoplasmic tail (42) was included as a negative control. This mutant glycoprotein, ERRS, is efficiently retrieved from the Golgi complex and, thus, is not processed to gp120 or expressed on the cell surface (47). Little or no ERRS gp160 could be detected with the biotinylation reagent (Fig. 5, Cell Surface, lane ERRS), despite the efficient expression of the glycoprotein intracellularly (Fig. 5, Cell Lysate, lane ERRS). Therefore, the assay was specific for proteins on the surface of the cell. Cell-surface immunofluorescent staining performed with the same subset of fusion-defective mutants was found to be consistent with the results presented here (data not shown). Thus, the effect of these mutations on fusion appears to be independent of the relative levels of glycoprotein on the cell surface.

**Expression of the envelope glycoprotein mutants in the context of virus.** The hydrophobic residues within the 17-amino-acid membrane-proximal region are all highly conserved, including the five tryptophan residues, which are present in every

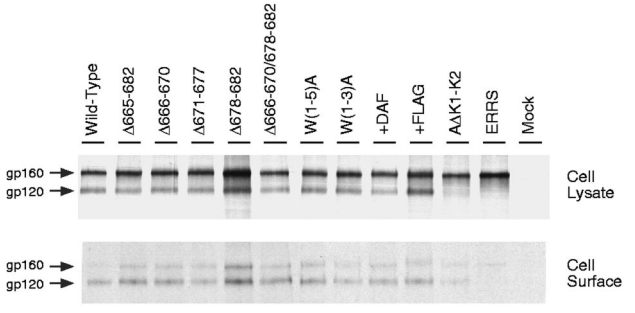


FIG. 5. Biotinylation of envelope glycoprotein mutants expressed on the cell surface. Proteins on the surface of metabolically radiolabeled glycoprotein-expressing cells were biotinylated with NHS-SS-biotin. Viral proteins were isolated by immunoprecipitation with patient serum, and a portion of the immunoprecipitate was analyzed by SDS-PAGE (top panel). The remaining immunoprecipitate was boiled to denature antibodies and incubated with streptavidin-agarose. Following washing, biotinylated proteins were released by reducing agent and analyzed by SDS-PAGE (8% polyacrylamide) (bottom panel). ERRS is a modified HIV-1 glycoprotein containing an endoplasmic reticulum retrieval signal attached to the C terminus of its cytoplasmic tail.

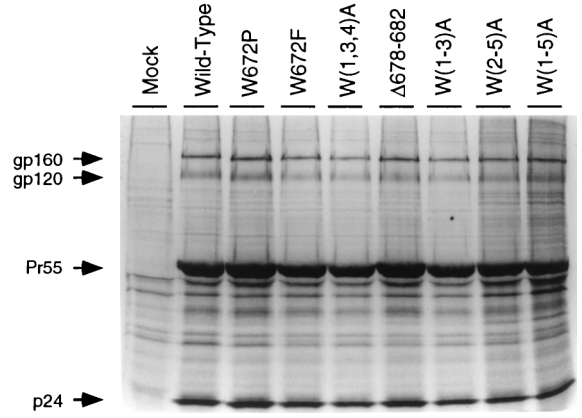


FIG. 6. Expression of envelope glycoprotein mutants in the context of virus. Radiolabeled viral proteins were immunoprecipitated from COS-1 cells expressing pNL4-3 proviral constructs by using patient serum and analyzed by SDS-PAGE (9% polyacrylamide). The positions of the $Pr55^{gag}$ and $p24^{gag}$ products are indicated to the left.

isolate of HIV-1 sequenced to date (Fig. 1A). Thus, it was surprising that only relatively severe mutations affecting these residues abrogated fusion in the cell-cell assay. In order to determine the effects of these mutations in a more biologically relevant functional assay, representative mutations were cloned into the pNL4-3 proviral construct for analysis of their effects on virus entry.

Figure 6 shows a pulse-chase analysis of viral protein expression for selected proviral constructs with various fusion phenotypes. All of the constructs express similar levels of normally processed glycoprotein, as well as Gag structural proteins, the Pr55 precursor and p24 capsid protein, compared to the levels of the wild-type construct. The other proviral constructs not depicted on this gel also expressed similar levels of properly processed viral proteins (data not shown).

**Effects of the mutations on virus entry.** To determine the effect of the mutations within the tryptophan-rich region on virus entry, virus was harvested from cells expressing the mutant proviral constructs and filtered to remove cellular debris. The relative levels of reverse transcriptase activity were determined for each of the virus stocks as a measure of the relative virus concentrations. The viral stocks were then normalized by appropriate dilution with media and used to infect MAGI cells. As with the cell-cell fusion assay above, the use of MAGI cells provides a highly sensitive assay for virus entry. Upon infection of the MAGI cell and expression of viral proteins, Tat induces expression of the β-galactosidase reporter gene. After staining in situ 48 h after virus adsorption, individual virus entry events can be scored by the presence of either single blue cells or blue syncytia, both containing intensely stained nuclei. Titration of virus stocks was shown to give linear results in this assay over a range of at least 3 logs, and in situ staining and microscopic quantitation have been shown to be much more sensitive than staining of cell lysates for colorimetric quantitation (31).

Under conditions in which approximately $10^4$ infectious units of wild-type virus was added to cells, it was possible to detect low levels of virus entry only with mutants which were permissive for fusion in the cell-cell assay (Fig. 7A). Two of these mutants, W672P and W672F, were dramatically more efficient at mediating entry than the other mutants. Yet even these mutants yielded only about 10% of the wild-type level of entry. Mutants which were markedly reduced in cell-cell fusion ability scored just barely above background levels of blue cells

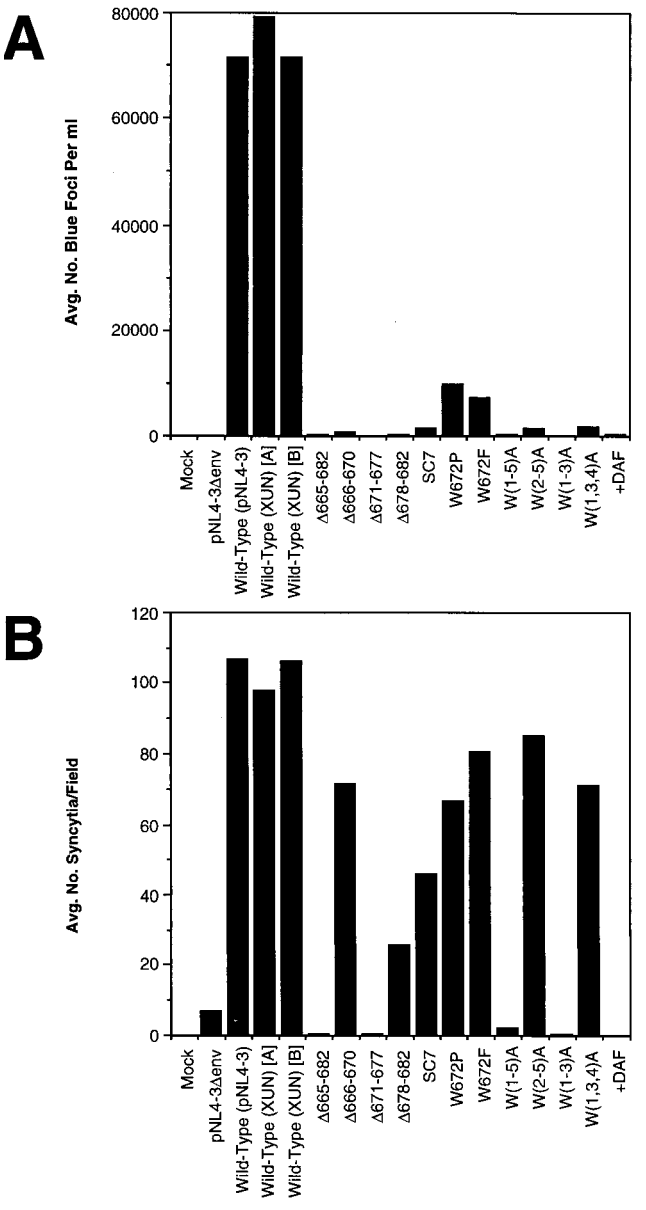


FIG. 7. Virus entry and cell-cell fusion in the context of virus. (A) Culture medium from cells expressing virus was filtered and normalized for reverse transcriptase activity. The normalized medium was used to infect duplicate wells (12-well plates) of HeLa-CD4/LTR-β-gal cells. Cells were stained with X-Gal in situ, and the numbers of blue foci (syncytia or single cells) were quantitated microscopically. Virus entry was quantitated as the average number of blue foci per milliliter by first counting the number of blue foci per 16-$mm^2$ field (low power) for six nonoverlapping fields in each of two wells. The average number of blue foci per field was then multiplied by the total number of fields per well, and the result was corrected for the volume of reverse transcriptase-normalized virus supernatant used. (B) Cell-cell fusion was determined as in the legend to Fig. 2, except that proviral constructs were expressed in 293T cells and mixed with HeLa-CD4/LTR-β-gal cells. Wild-Type (XUN) [A] and Wild-Type (XUN) [B] indicate results from two separately prepared pNL4-3/XUN plasmid clones. pNL4-3Δenv, pNL4-3 containing a frameshift mutation in gp120.

(Fig. 7A, Δ666–670 and Δ678–682). Since these results did not parallel the results of the cell-cell fusion assay, it was important to determine whether these proviral constructs were capable of mediating cell-cell fusion comparably to the glycoprotein expression constructs. When cells expressing each of the viral

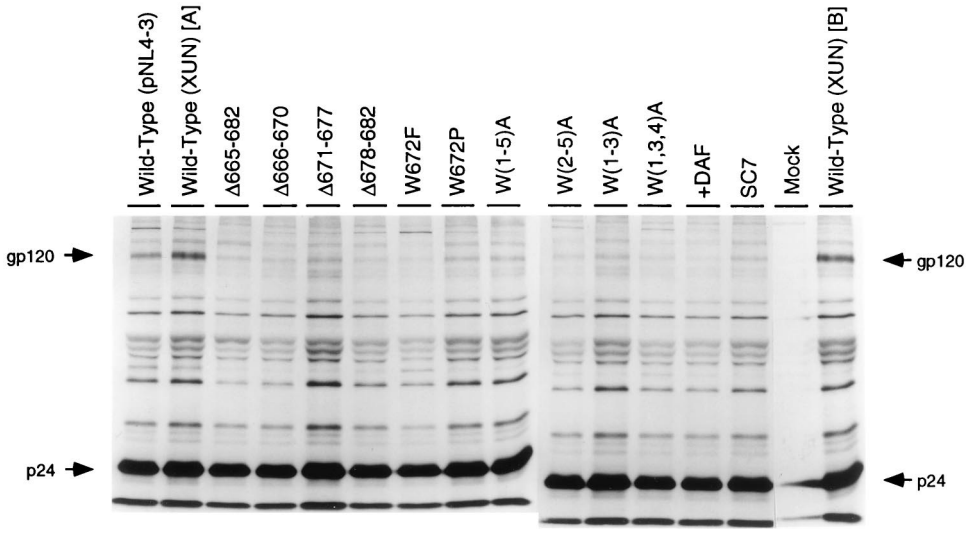


FIG. 8. Incorporation of envelope glycoprotein mutants into virions. Supernatants were collected from metabolically radiolabeled 293T cells expressing pNL4-3 proviral constructs and filtered to remove cellular debris. Virus was pelleted from the filtered supernatants through a sucrose cushion and solubilized in lysis buffer. Viral proteins were immunoprecipitated and analyzed by SDS-PAGE (9% polyacrylamide).

constructs were mixed 1:10 with MAGI cells, the levels of fusion seen with each construct paralleled the results of the COS-1 cell-mediated fusion assays (compare Fig. 7B with Fig. 2). Moreover, in the virus entry assays, those mutant constructs which showed limited infectivity and which were permissive in the cell-cell fusion assay yielded small syncytia of a size similar to those seen with the wild-type clones (data not shown). These results demonstrate that the dramatic decrease in virus entry seen with the permissive mutant viral constructs was not due to a general defect in fusion, but rather to a specific inhibition of virus entry.

**Incorporation of the envelope glycoprotein mutants into virions.** In order to determine whether defects in infectivity reflected a difference in the efficiency with which the mutant glycoproteins were incorporated into virions, the composition of released virions was assessed following metabolic labeling. Supernatants were collected from cells expressing the proviral constructs and filtered to remove cellular debris. Virus was pelleted from the supernatants through a sucrose cushion and solubilized for immunoprecipitation and SDS-PAGE. As can be seen in Fig. 8, the mutant glycoproteins were all detected in the viral pellets at levels much lower than those of wild-type glycoprotein. This demonstrates that even subtle changes in the tryptophan-rich region can dramatically affect incorporation of glycoprotein into virions.

## DISCUSSION

The region proximal to the N-terminal border of the membrane-spanning domain of the HIV-1 glycoprotein is unusually rich in tryptophan residues, containing five that are conserved in all sequenced strains of HIV-1, and is predicted by computer algorithms to form an α-helix with a partial amphipathic character (Fig. 1C). Three of these tryptophan residues, the first, third, and fourth in this region, are also completely conserved among sequenced strains of HIV-2 and SIV (Fig. 1B). Two of the tryptophan residues align with the analogous region in the visna virus lentiviral glycoprotein, and a third is present within the membrane-spanning domain of this protein. In addition to the tryptophan residues, the other hydrophobic residues in this region are also very highly conserved among different strains of HIV-1 (Fig. 1A). This region of the HIV-1 glycoprotein also overlaps the C-terminal sequence of a peptide, DP-178, which has been shown to potently inhibit fusion and virus entry (61), as well as the epitope for a neutralizing human monoclonal antibody, 2F5 (Fig. 1A). These factors suggest an important role for the membrane-proximal region in the function of lentiviral glycoproteins.

All of the mutated Env proteins were expressed at levels comparable to those of wild-type Env and, except for AΔK1-K2, were transported and processed normally. This suggests that all of these proteins folded in a manner similar to that of wild-type Env, since multiple studies suggest that misfolded oligomeric membrane glycoproteins tend not to be transported through the normal secretory pathway but are, rather, retained in the endoplasmic reticulum and degraded intracellularly (14). In addition, Env proteins with mutations which resulted in reduced levels of fusion were maintained on the cell surface at steady-state levels comparable to those of wild-type Env. Thus, the decreased levels of fusion seen with these mutants do not appear to be due to lower concentrations of Env on the cell surface. Finally, mutated Env proteins exhibiting reduced levels of fusion were capable of binding CD4 as efficiently as wild-type Env, indicating that the effect on fusion was independent of primary receptor binding.

Previous analyses by successive truncations of gp41 have demonstrated that amino acid residues 666 to 682 of the HIV-1 transmembrane glycoprotein are not sufficient to anchor the protein in the membrane (17, 43). Our results from the deletion of this region (mutant Δ665–682) demonstrate further that this region is also not necessary for the stable anchoring of the envelope glycoprotein complex in the plasma membrane. This is consistent with lysine 683 forming the amino-terminal border of the membrane-spanning domain. Our results also demonstrate that residues 666 to 682 are not necessary for the transport and processing or CD4-binding capability of the envelope glycoprotein.

A deletion of 11 residues, which included the entire 2F5 epitope and which overlapped the DP-178 and tryptophan-rich regions, has been shown previously to abrogate fusion (44). This deletion overlapped the site of the +FLAG insertion, which we demonstrate also abrogated fusion. However, since

our Δ666–670 mutation did not abrogate fusion, the effect of the previously reported 11-residue deletion appears not to have been due solely to the deletion of residues within the tryptophan-rich region.

The mutant AK2 is similar to a mutant reported by Helseth et al. in which the lysine which forms the amino-terminal border of the membrane-spanning domain was substituted for by an isoleucine (26). However, unlike that mutant, which essentially abrogated fusion, AK2, which substitutes three amino acids including an asparagine for the membrane-bordering lysine, maintained a significant level of fusogenicity (about 30% of the wild-type level). Since substitution of the hydrophobic residues at positions 684 and 685, which were also substituted in the AK2 mutation, had been shown previously not to affect fusion (20), it is likely that substitution of Lys 683 is responsible for the effect of the Helseth mutation. Perhaps the polarity of the asparagine in this position in the AK2 mutant, together with the adjacent serine, preserves the functionality of this region.

The central seven amino acid residues in the tryptophan-rich region, 671 to 677, appear to be critical for the cell-cell fusion activity of the transmembrane glycoprotein. The reduction of the fusogenicity of the glycoprotein by scrambling these central residues (mutant SC7) suggests that the clustering of hydrophobic residues in an α-helical secondary structure as modeled in Fig. 1C may indeed be important, but not essential, for the function of this region. The mutation ΔFN was found to have no significant effect on fusion. This result would argue against a topological requirement for a helical structure, but it does not necessarily preclude the existence of such a structure. Indeed, when the mutated sequence was modeled as a helical net, the conserved hydrophobic residues still tended to cluster together on one face of the helix, although in a somewhat distorted arrangement (data not shown). The lack of major effect caused by the proline substitution for the central tryptophan (W672P) further suggests that a rigid helical structure in this region is not required, although again this does not preclude the existence of a relatively refractory helical structure. Indeed, in the context of membranes, proline residues have been found to stabilize α-helical secondary structure, while disrupting β-sheet structures (33).

Residues 666 to 670 and 678 to 682 also appear to affect fusion, because deletion of these residues reduced, but did not block, cell-cell fusion. Our results suggest that the specific amino acid sequence of residues 665 to 667 is not critical for cell-cell fusion, since the AK1 mutant was fusogenic. Therefore, it is unlikely that the epitope for the neutralizing monoclonal antibody 2F5 which overlaps these residues is involved in a specific molecular interaction during the cell-cell fusion event. Since the epitope for 2F5 is accessible to antibody, it seemed possible that this epitope might accommodate the additional hydrophilic residues added in the +FLAG mutation and perhaps allow the use of the commercially available anti-FLAG M2 monoclonal antibody (Kodak-IBI) to neutralize virus. However, the +FLAG mutation blocked fusion, perhaps by acting in an analogous fashion to the bound antibody in preventing fusion.

Recent results from structural analyses of gp41 suggest that the inhibitory peptide, DP-178, might act by competing for a binding groove (for a C-terminal heptad repeat) on a coiled-coil trimer formed from an amino-terminal heptad repeat (7, 56). Since Δ666–670 maintained a reduced level of fusogenicity, it seems likely that the C-terminal portion of DP-178, which overlaps residues 665 to 673, is not absolutely required for the formation of a stable six-helix coiled-coil bundle. However, in an analysis of the inhibitory potential of truncated DP-178 peptides, this overlapping region appeared to be important for the inhibitory function of DP-178 (59). One might expect that DP-178 would inhibit fusion of glycoproteins with mutations in this region as well as, if not better than, the wild-type glycoprotein, since it would now be competing with a truncated C-terminal heptad repeat. Mutants AK1, W(2,3)A, and W666A/W672S were therefore tested for their ability to be inhibited by DP-178 in the cell-cell fusion assay, and all were inhibited completely by the peptide, as was the wild-type protein (data not shown). Recent studies with more sensitive fusion assays indicate that such mutants are indeed more susceptible to inhibition by DP-178 (39).

Although the tryptophan-rich region is predicted to be α-helical, modeling the region as a β-strand also positions all five tryptophans on the same side of the structure (data not shown). However, in this case, the conserved hydrophobic residues are interspersed with the nonconserved polar residues. Interestingly, the structure of the fusion peptide of gp41 from peptide studies appears to be a β-strand until it contacts the lipid bilayer, at which time it transitions to a partly α-helical structure (36). Thus, the tryptophan-rich region, which has similar characteristics to the fusion peptide and could interact with membranes, may also undergo a conformational change involving the conserved tryptophan residues. In support of this hypothesis, the 2F5 epitope which overlaps the N-terminal end of this region can no longer be recognized by antibody following CD4 binding (51), suggesting that this region may indeed undergo a conformational change or become sequestered in the membrane during fusion. Thus, fusion-inhibitory mutations within the tryptophan-rich domain may interfere with a late step in membrane fusion, perhaps involving an interaction with, and possibly the disruption of, the membrane in which the glycoprotein is anchored.

Substitution of alanines or other amino acids for the individual conserved tryptophan residues had no significant effect on cell-cell fusion. It is important to note, however, that the overnight cell-cell fusion assay employed here may not reveal subtle variations in the kinetics of the fusion reaction among different mutated proteins. Results from mutations combining multiple tryptophan substitutions demonstrate both the unusual resiliency and intricacy of the function of this region during fusion. The fact that W(1–5)A and W(1–3)A both abrogated syncytium formation suggested that the first three tryptophans were critical for the function of this region, but other combinations of mutations have failed to pinpoint any particular tryptophan residue as critical for the function of this domain. Indeed, recent studies suggest that even the W(1–5)A mutant can induce the formation of small fusion pores but that these are unable to be propagated into syncytia (39).

A striking feature of many of the mutations analyzed was the propensity to inhibit virus entry to levels at the limit of detection, while having only a limited effect on syncytium formation. For example, the W672P and W672F mutants exhibited less than a 20% reduction in fusogenicity compared to wild-type Env, yet reduced the number of virus entry events by almost 90% compared to the wild type. The most likely explanation for this dramatic effect on virus entry is that the reduced concentration of Env protein on the surface of the virion is below a critical concentration required for virus-cell fusion. In contrast, the high concentration of Env protein on the cell surface may facilitate fusion, overcoming a block to fusion which occurs in the context of virus, perhaps by enhancing the recruitment of Env oligomers into fusion pores. Studies with influenza virus HA have demonstrated that variations in the cell surface concentration of fusogenic protein oligomers correspond directly with the efficiency of fusion pore formation

(11). W672F and W672P, however, were much more efficient at mediating virus entry relative to the other mutants, even though cell-cell fusion efficiencies and the reduced levels of incorporation appeared similar. This discrepancy could indicate that the virus entry assay is able to discriminate more subtle differences in fusogenicity than the cell-cell assay. It does indicate that these mutants are less defective for fusion in the context of the virus than the other mutants tested.

It is clear from the striking effect of a conservative single amino acid substitution (W672F) on incorporation of glycoprotein into virions and virus entry that the function of this region in the virus life cycle is dependent specifically upon the tryptophan residues which lie within it, rather than simply on the hydrophobic moment of the domain or the presence of an aromatic side chain. This result may explain the absolute conservation of these residues among all strains of HIV-1. Tryptophan residues have been suspected to play an important role in a number of biologic processes (2). One possible role for the conserved tryptophan residues in enhancing the incorporation of glycoprotein into virions could involve an interaction with components of the membrane, such as cholesterol (12). Such interactions could facilitate the enrichment of glycoprotein within certain subdomains of the plasma membrane (4) through which the virus might bud. HIV does contain a higher cholesterol/phospholipid ratio in its viral membrane than is found in most cell membranes (1). An interaction with cholesterol in the target cell membrane might also facilitate membrane fusion. Interestingly, the presence and positioning of tryptophan residues in the bee venom peptide melittin have been shown to be critical for its hemolytic activity (2). Thus, the conserved tryptophan residues could interact with cholesterol in either the membrane in which the glycoprotein is anchored, the target cell membrane, or both. The effects of these mutations within the tryptophan-rich region of HIV-1 Env are similar to those found upon mutagenesis of an analogous region of the Newcastle disease virus fusion protein, which was also relatively refractory to single amino acid substitutions but sensitive to multiple, combined substitutions (45). This region of the Newcastle disease virus fusion protein contains a leucine zipper-like motif and was also hypothesized to interact with membranes during fusion because of its proximity to the membrane.

In an earlier mutational analysis of this region, Cao et al. (6) concluded that the single amino acid substitution W672P resulted in Env proteins which were up to twice as fusogenic as wild-type Env in a syncytium assay. Cell-cell fusion was quantitated by counting the number of syncytia per unit of area in a transfected T-cell culture. We were unable to duplicate these results in syncytium assays using adherent cells. Cao et al. also reported that the effect of W672P and other single amino acid substitutions within this region on virus entry was minimal. Virus entry into T cells was determined by using an *env* transcomplementation system (25), and complemented virus was produced by cotransfection of an *env* expression plasmid with an *env*-defective provirus containing a chloramphenicol acetyltransferase (CAT) reporter gene. Relative levels of entry were determined by measuring the CAT activity in the lysates of T cells following incubation with virus. In a complementation assay such as this, it may be possible to "overload" virions with more Env protein than would normally be incorporated into virus expressed from a complete genome. If such were the case, then the complementation assay might be expected to underestimate the detrimental effects of subtle mutations on virus replication in much the same way as a syncytium assay may underestimate a mutation's effect on membrane fusion. This may explain why the same mutation was much more inhibitory in the context of an intact provirus from our analysis than it was in the complementation system from the previous study.

It is unclear at present how this region, and the conserved tryptophan residues in particular, are functioning in the membrane fusion mechanism. It is also unclear how even conservative mutations within this tryptophan-rich motif dramatically reduced the efficiency with which glycoprotein was incorporated into virions. Interestingly, however, mutations within the proteolytic cleavage site of gp160 have also been shown to reduce glycoprotein incorporation (15), suggesting that the conformation of the glycoprotein ectodomain can affect molecular interactions during virus assembly.

## ACKNOWLEDGMENTS

We thank Mark Mulligan (University of Alabama at Birmingham) for providing CD4-IgG (Genentech) and Eric Freed (National Institutes of Health) for providing the pNL4-3 construct.

This work was supported by research grant AI-33319 from the National Institutes of Health. Virus culture was performed in the University of Alabama at Birmingham Center For AIDS Research Central Virus Core Facility, supported by program grant AI-27767 from the National Institutes of Health. K. Salzwedel was supported in part by training grant CA-09467 from the National Institutes of Health.

## REFERENCES

1. **Aloia, R. C., C. C. Curtain, and F. C. Jensen.** 1992. Membrane cholesterol and human immunodeficiency virus infectivity, p. 283–303. *In* R. C. Aloia and C. C. Curtain (ed.), Membrane interactions of HIV: implications for pathogenesis and therapy in AIDS, vol. 6. Wiley-Liss, Inc., New York, N.Y.
2. **Blondelle, S. E., L. R. Simpkins, E. Perez-Paya, and R. A. Houghton.** 1993. Influence of tryptophan residues on melittin's hemolytic activity. Biochim. Biophys. Acta **1202:**331–336.
3. **Bosch, M. L., P. L. Earl, K. Fargnoli, S. Picciafuoco, F. Giombini, F. Wong-Staal, and G. Franchini.** 1989. Identification of the fusion peptide of primate immunodeficiency viruses. Science **244:**694–697.
4. **Brown, D., and E. London.** 1997. Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? Biochem. Biophys. Res. Commun. **240:**1–7.
5. **Bullough, P. A., F. M. Hughson, J. J. Skehel, and D. C. Wiley.** 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. Nature (London) **371:**37–43.
6. **Cao, J., L. Bergeron, E. Helseth, M. Thali, H. Repke, and J. Sodroski.** 1993. Effects of amino acid changes in the extracellular domain of the human immunodeficiency virus type 1 gp41 envelope glycoprotein. J. Virol. **67:**2747–2755.
7. **Chan, D., D. Fass, J. Berger, and P. Kim.** 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell **89:**263–273.
8. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **17:**2745–2752.
9. **Chen, S. S.** 1994. Functional role of the zipper motif region of human immunodeficiency virus type 1 transmembrane protein gp41. J. Virol. **68:**2002–2010.
10. **Chen, S. S.-L., C.-N. Lee, W.-R. Lee, K. McIntosh, and T.-H. Lee.** 1993. Mutational analysis of the leucine zipper-like motif of the human immunodeficiency virus type 1 envelope transmembrane glycoprotein. J. Virol. **67:**3615–3619.
11. **Danieli, T., S. L. Pelletier, Y. I. Henis, and J. M. White.** 1996. Membrane fusion mediated by the influenza virus hemagglutinin requires the concerted action of at least three hemagglutinin trimers. J. Cell Biol. **133:**559–569.
12. **DeKruijff, B.** 1990. Cholesterol as a target for toxins. Biosci. Rep. **10:**127–130.
13. **Doms, R. W.** 1993. Protein conformational changes in virus-cell fusion. Methods Enzymol. **221:**61–72.
14. **Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius.** 1993. Folding and assembly of viral membrane proteins. Virology **193:**545–562.
15. **Dubay, J. W., S. R. Dubay, H.-J. Shin, and E. Hunter.** 1995. Analysis of the cleavage site of the human immunodeficiency virus type 1 glycoprotein: requirement of precursor cleavage for glycoprotein incorporation. J. Virol. **69:**4675–4682.
16. **Dubay, J. W., S. J. Roberts, B. Brody, and E. Hunter.** 1992. Mutations in the leucine zipper of the human immunodeficiency virus type 1 transmembrane glycoprotein affect fusion and infectivity. J. Virol. **66:**4748–4756.
17. **Dubay, J. W., S. J. Roberts, B. H. Hahn, and E. Hunter.** 1992. Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity. J. Virol. **66:**6616–6625.

18. **Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane G protein-coupled receptor. Science **272:**872–877.
19. **Freed, E. O., D. J. Myers, and R. Risser.** 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. Proc. Natl. Acad. Sci. USA **87:**4650–4654.
20. **Gabuzda, D., U. Olshevsky, P. Bertani, W. A. Haseltine, and J. Sodroski.** 1991. Identification of membrane anchorage domains of the HIV-1 gp160 envelope glycoprotein precursor. J. Acquired Immune Defic. Syndr. **4:**34–40.
21. **Gabuzda, D. H., A. Lever, E. Terwilliger, and J. Sodroski.** 1992. Effects of deletions in the cytoplasmic domain on biological functions of human immunodeficiency virus type 1 envelope glycoproteins. J. Virol. **66:**3306–3315.
22. **Gallaher, W. R.** 1987. Detection of a fusion peptide sequence in the transmembrane protein of the human immunodeficiency virus. Cell **50:**327–328.
23. **Gaudin, Y., R. W. H. Ruigrok, and J. Brunner.** 1995. Low-pH induced conformational changes in viral fusion proteins: implications for the fusion mechanism. J. Gen. Virol. **76:**1541–1556.
24. **Gilbert, J. M., L. D. Hernandez, J. W. Balliet, P. Bates, and J. M. White.** 1995. Receptor-induced conformational changes in the subgroup A avian leukosis and sarcoma virus envelope glycoprotein. J. Virol. **69:**7410–7415.
25. **Helseth, E., M. Kowalski, D. Gabuzda, U. Olshevsky, W. Haseltine, and J. Sodroski.** 1990. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. J. Virol. **64:**2416–2420.
26. **Helseth, E., U. Olshevsky, D. Gabuzda, B. Ardman, W. Haseltine, and J. Sodroski.** 1990. Changes in the transmembrane region of the human immunodeficiency virus type 1 gp41 envelope glycoprotein affect membrane fusion. J. Virol. **64:**6314–6318.
27. **Hernandez, L. D., and J. M. White.** Personal communication.
28. **Jiang, S., K. Lin, N. Strick, and A. R. Neurath.** 1993. HIV-1 inhibition by a peptide. Nature (London) **365:**113.
29. **Johnston, P. B., and E. Hunter.** Unpublished results.
30. **Kemble, G. W., T. Danieli, and J. M. White.** 1994. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. Cell **76:**383–391.
31. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66:** 2232–2239.
32. **Lee, W.-R., X.-F. Yu, W.-J. Syu, M. Essex, and T.-H. Lee.** 1992. Mutational analysis of conserved N-linked glycosylation sites of human immunodeficiency virus type 1 gp41. J. Virol. **66:**1799–1803.
33. **Li, S.-C., N. K. Goto, K. A. Williams, and C. M. Deber.** 1996. α-Helical, but not β-sheet, propensity of proline is determined by peptide environment. Proc. Natl. Acad. Sci. USA **93:**6676–6681.
34. **Lisanti, M. P., M. Sargiacomo, L. Graeve, A. R. Saltiel, and E. Rodriguez-Boulan.** 1988. Polarized apical distribution of glycosyl-phosphatidylinositol-anchored proteins in a renal epithelial cell line. Proc. Natl. Acad. Sci. USA **85:**9557–9561.
35. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Adv. Virus Res. **36:**107–151.
36. **Martin, I., H. Schaal, A. Scheid, and J.-M. Ruysschaert.** 1996. Lipid membrane fusion induced by the human immunodeficiency virus type 1 gp41 N-terminal extremity is determined by its orientation in the lipid bilayer. J. Virol. **70:**298–304.
37. **McClure, M. O., M. Marsh, and R. A. Weiss.** 1988. Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. EMBO J. **7:**513–518.
38. **McClure, M. O., M. A. Sommerfelt, M. Marsh, and R. A. Weiss.** 1990. The pH independence of mammalian retrovirus infection. J. Gen. Virol. **71:**767–773.
39. **Muñoz-Barroso, I., K. Salzwedel, E. Hunter, and R. Blumenthal.** Unpublished data.
40. **Muster, T., R. Guinea, A. Trkola, M. Purtscher, A. Klima, F. Steindl, P. Palese, and H. Katinger.** 1994. Cross-neutralizing activity against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. J. Virol. **68:**4031–4034.
41. **Muster, T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, F. Rüker, and H. Katinger.** 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J. Virol. **67:**6642–6647.
42. **Nilsson, T., and G. Warren.** 1994. Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus. Curr. Opin. Cell Biol. **6:**517–521.
43. **Owens, R. J., C. Burke, and J. K. Rose.** 1994. Mutations in the membrane-spanning domain of the human immunodeficiency virus envelope glycoprotein that affect fusion activity. J. Virol. **68:**570–574.
44. **Poumbourios, P., W. El Ahmar, D. A. McPhee, and B. E. Kemp.** 1995. Determinants of human immunodeficiency virus type 1 envelope glycoprotein oligomeric structure. J. Virol. **69:**1209–1218.
45. **Reitter, J. N., T. Sergel, and T. G. Morrison.** 1995. Mutational analysis of the leucine zipper motif in the Newcastle disease virus fusion protein. J. Virol. **69:**5995–6004.
46. **Salzwedel, K., P. B. Johnston, S. J. Roberts, J. W. Dubay, and E. Hunter.** 1993. Expression and characterization of glycophospholipid-anchored human immunodeficiency virus type 1 envelope glycoproteins. J. Virol. **67:** 5279–5288.
47. **Salzwedel, K., J. T. West, Jr., M. J. Mulligan, and E. Hunter.** 1998. Retention of the human immunodeficiency virus type 1 envelope glycoprotein in the endoplasmic reticulum does not redirect virus assembly from the plasma membrane. J. Virol. **72:**7523–7531.
48. **Sattentau, Q. J., and J. P. Moore.** 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J. Exp. Med. **174:**407–415.
49. **Sattentau, Q. J., and J. P. Moore.** 1993. The role of CD4 in HIV binding and entry. Phil. Trans. R. Soc. Lond. B Biol. Sci. **342:**59–66.
50. **Sattentau, Q. J., J. P. Moore, F. Vignaux, F. Traincard, and P. Poignard.** 1993. Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding. J. Virol. **67:**7383–7393.
51. **Sattentau, Q. J., S. Zolla-Pazner, and P. Poignard.** 1995. Epitope exposure on functional, oligomeric HIV-1 gp41 molecules. Virology **206:**713–717.
52. **Steffy, K. R., G. Kraus, D. J. Looney, and F. Wong-Staal.** 1992. Role of the fusogenic peptide sequence in syncytium induction and infectivity of human immunodeficiency virus type 2. J. Virol. **66:**4532–4535.
53. **Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman.** 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell **49:**659–668.
54. **Weiss, C. D., and J. M. White.** 1993. Characterization of stable Chinese hamster ovary cells expressing wild-type, secreted, and glycosylphosphatidylinositol-anchored human immunodeficiency virus type 1 envelope glycoprotein. J. Virol. **67:**7060–7066.
55. **Weissenhorn, W., L. Calder, A. Dessen, T. Laue, J. Skehel, and D. Wiley.** 1997. Assembly of a rod-shaped chimera of a trimeric GCN4 zipper and the HIV-1 gp41 ectodomain expressed in Escherichia coli. Proc. Natl. Acad. Sci. USA **94:**6065–6069.
56. **Weissenhorn, W., A. Dessen, S. Harrison, J. Skehel, and D. Wiley.** 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature **387:**426–430.
57. **Weissenhorn, W., S. Wharton, L. Calder, P. Earl, B. Moss, E. Aliprandis, J. Skehel, and D. Wiley.** 1996. The ectodomain of HIV-1 env subunit gp41 forms a soluble, alpha-helical, rod-like oligomer in the absence of gp120 and the N-terminal fusion peptide. EMBO J. **15:**1507–1514.
58. **Wild, C., J. W. Dubay, T. Greenwell, T. Baird, Jr., T. G. Oas, C. McDanal, E. Hunter, and T. Matthews.** 1994. Propensity for a leucine zipper-like domain of human immunodeficiency virus type 1 gp41 to form oligomers correlates with a role in virus-induced fusion rather than assembly of the glycoprotein complex. Proc. Natl. Acad. Sci. USA **91:**12676–12680.
59. **Wild, C., T. Greenwell, D. Shugars, L. Rimsky-Clarke, and T. Matthews.** 1995. The inhibitory activity of an HIV type 1 peptide correlates with its ability to interact with a leucine zipper structure. AIDS Res. Hum. Retroviruses **11:**323–325. (Letter to the editor.)
60. **Wild, C., T. Oas, C. McDanal, D. Bolognesi, and T. Matthews.** 1992. A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. Proc. Natl. Acad. Sci. USA **89:**10537–10541.
61. **Wild, C. T., D. C. Shugars, T. K. Greenwell, C. B. McDanal, and T. J. Matthews.** 1994. Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. Proc. Natl. Acad. Sci. USA **91:**9770–9774.
62. **Wilk, T., T. Pfeiffer, and V. Bosch.** 1992. Retained in vitro infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the env gene product. Virology **189:**167–177.
63. **Yu, X., X. Yuan, M. F. McLane, T.-H. Lee, and M. Essex.** 1993. Mutations in the cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein impair the incorporation of Env proteins into mature virions. J. Virol. **67:**213–221.